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Review Article

Amplification and Subtraction Methods and Their Application to the Discovery of Novel Human Viruses

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INTRODUCTION

Since the first experiment describing the cloning of foreign DNA in a bacterial plasmid [Cohen et al., 1973] there have been as many genes isolated, characterized, and sequenced from a plethora of organisms as there have been modifications to the original cloning methods. These modifications and improvements have included the construction and engineering of plasmid and viral vectors for the efficient cloning and/or expression of foreign genes; the generation of cell lines with very high transformation efficiencies for the production of recombinant libraries; and the development of sophisticated subtraction techniques for the isolation of rare or differentially expressed genes, to name a few. However, with the advent of the polymerase chain reaction (PCR) and thermostable DNA polymerase [Saiki et al., 1988], there has been a dramatic increase in the development of methods designed to isolate genes of interest and also in the number of genes that have actually been isolated and characterized. These PCR-based methods have been employed for the elucidation of eukaryotic and prokaryotic genes, as well as in the isolation of sequences from known and novel viruses. It is the purpose of this review to examine some of the amplification-based and subtractive methods that have been utilized for the isolation of novel viruses that cause human diseases. These methods include nonspecific amplification of cDNAs (NSA) coupled with immunoscreening, representational difference analysis (RDA), and genomic mismatch scanning (GMS).

Non-specific Amplification Methods and Immunoscreening of cDNA Libraries

The construction of cDNA and genomic DNA libraries in either bacterial plasmids or phage lambda has become a standard method in molecular biology for the isolation of novel gene sequences [Gubler and Hoffman, 1983; Sambrook et al., 1989]. These libraries are screened using nucleic acid probes—either cDNAs, oligonucleotides, or gene fragments [Benton and Davis, 1977]. When expression libraries are constructed, the resulting recombinants are screened immunologically using antisera prepared against a purified protein [Young and Davis, 1983a; Young and Davis, 1983b], or in the case of viral infection, using serum from an individual infected with the putative etiologic agent. These standard techniques generally require at least 0.20–1.0 μg of mRNA in order to generate a library with 10^5–10^6 recombinants. However, recent developments in amplification technologies have made the construction of representative libraries—in particular, cDNA libraries—much more efficient, whereby the resultant cDNA library will possess nearly all nucleic acid sequences representative of the source organism. Several of the amplification methods utilized in cDNA library construction are discussed in this review.

Non-specific Amplification (NSA) or Sequence-independent Single Primer Amplification (SISPA)

This relatively simple method is used for the nonspecific amplification of DNA molecules (Fig. 1). The technique does not require prior knowledge of the target sequence and is particularly useful when the target is present in extremely low concentrations or when the amount of starting material is highly limited. The method utilizes standard methods of cDNA synthesis followed by the blunt-end ligation of an annealed primer pair to each end of the cDNA molecule. The earliest report on this technique, referred to as primer-directed enzymatic amplification [Akowitz and Manue-

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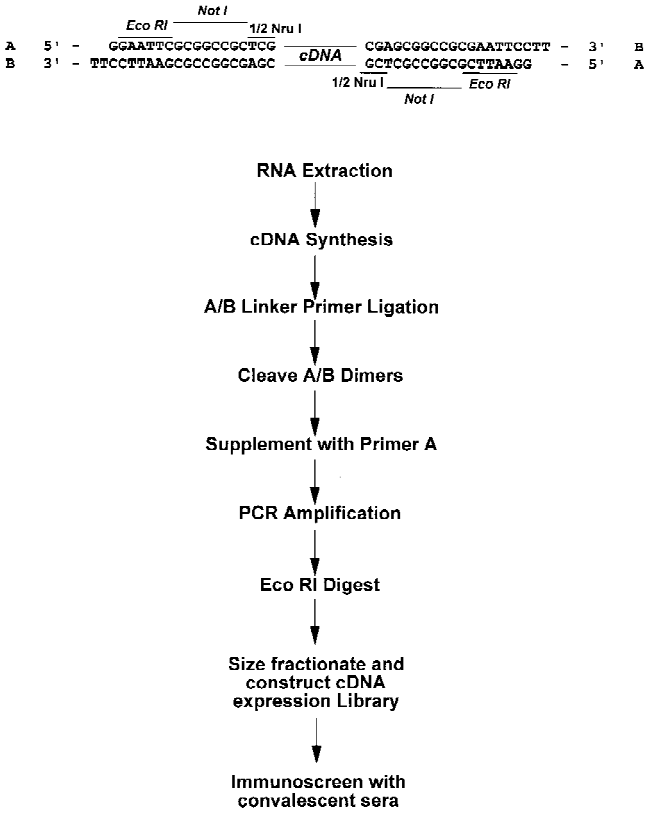

Fig. 1. SISPA linker/primer sequence and its use in cDNA library construction. The A/B linker/primer pair shown possesses two restriction sites (EcoRI and NotI) to facilitate cloning of amplified cDNAs into a suitable vector and also has an asymmetric structure at its 3′-end to ensure directional ligation to blunt-ended cDNAs. The partial NruI site is reformed upon ligation of a linker/primer pair; these molecules can be removed from the ligation products by digestion with NruI. An experimental approach for utilization of the A/B linker/primers is outlined [Reyes and Kim, 1991].

lidis, 1989], demonstrated the production of a highly representative λgt10 cDNA library from as little as 20 pg of globin mRNA. Other investigators have utilized successfully very similar methods for the construction of cDNA libraries [Belyavsky et al., 1989; Frohman et al., 1988] for the amplification of cloned cDNAs [Rasmussen et al., 1989] or the amplification of genomic DNA sequences [Kinzler and Vogelstein, 1989]. A subsequent embodiment of this procedure demonstrated the efficient ligation of annealed primer pairs to, and subsequent amplification of, as little as 10 fg of HaeIII digested phi X174 DNA [Reyes and Kim, 1991].

This nonspecific amplification method has been applied to the isolation of novel viral sequences from infectious sources. Matsui et al. [1991] isolated an immunoreactive region of a Norwalk virus genome from a λgt11 cDNA library that was immunoscreened with antiserum from a convalescent patient. The Norwalk-like viruses are small, round-structured viruses [Greenberg et al., 1989] implicated in approximately 65% of acute nonbacterial gastroenteritis in the United States [Blacklow and Cukor, 1982]. Matsui and coworkers [1991] prepared cDNAs from 10 μg of nucleic acid isolated from 1.5 grams of an infectious stool sample estimated to contain 10^5–10^6 virion particle per gram. The cDNAs were subsequently amplified using the SISPA technique [Reyes and Kim, 1991] prior to cloning into λgt11. The immunoscreening of 120,000 recombinants resulted in the isolation of six overlapping clones. The sequence of these cDNAs was shown to be derived from the RNA genome of a Norwalk virus based on absence of sequence identity with known viruses, a lack of immunoreactivity with plasma from uninfected individuals, specific hybridization to RNA extracted from post-infection but not pre-infection stool samples, and hybridization only to nucleic acids isolated from cesium chloride density gradient fractionated infectious material. SISPA was also applied successfully to the isolation of immunogenic regions encoded by the genome of human astrovirus serotype-1 [Matsui et al., 1993]. Thus the utilization of this amplification technique allowed the isolation of immunogenic regions of the genome of infectious agents that had eluded classical cloning efforts. Another example of the successful application of this technique was demonstrated by Reyes et al. [1990], who isolated and characterized the hepatitis E virus (HEV). Although SISPA was not used to isolate sequences from the HEV RNA genome, it was used to increase the sensitivity of HEV RNA detection in infectious samples by southern hybridization of SISPA-amplified cDNAs. Nonspecific amplification has also been applied to the isolation of sequences derived from the hepatitis C virus (HCV) genome [Inchauspe et al., 1991]. These investigators used immunoaffinity chromatography to concentrate HCV viral particles from 100 ml of infectious plasma containing approximately 10^6 infectious doses/ml prior to nucleic acid extraction and cDNA synthesis. The cDNA population was subsequently amplified nonspecifically and then cloned into the Lambda-ZAP vector. The resultant library, constructed from 1 μg of amplified cDNA, contained 10 million recombinants from which three clones were isolated following hybridization to radiolabeled oligonucleotide probes complementary to the HCV sequence.

A technique similar to SISPA or NSA has been reported by Froussard [1992]. This method, referred to as random PCR (rPCR), utilizes an oligonucleotide primer possessing a 20 nucleotide "universal" sequence (containing restriction enzyme recognition sites) followed by a random hexamer at the 3′-end. This primer is used for both first and second strand cDNA synthesis. PCR amplification of the cDNAs is carried out using the universal region of the primer. This method obviates the inefficient blunt-end ligation of the linker/primer used in SISPA to the cDNAs ends. Froussard [1992] was able to demonstrate the efficient amplification of as little as 0.1 fg of MS2 phage RNA. This method is at least 100-fold more efficient than the SISPA method [Reyes and Kim, 1991]. A method very similar to this was used by Hayashi et al. [1993] to isolate nearly the entire genome of a Japanese isolate of HCV. These authors used essentially the same method as that de-

scribed by Froussard [1992] to generate cDNAs from RNA isolated from 2 ml of patient's serum. A second round of PCR using hemi-nested primers was used to amplify HCV-specific sequences.

The NSA technique has been utilized recently to identify immunogenic regions of a novel hepatitis agent, GB virus B [Pilot-Matias et al., 1996]. The GB agent was initially described by Deinhardt et al. [1967] and originated from the serum of a 34-year-old surgeon (with the initials GB) obtained during the third day of jaundice. Passage studies in nonhuman primates [Deinhardt et al., 1975; Feinstone et al., 1981; Holmes et al., 1973; Karayiannis et al., 1989; Tabor et al., 1979; Tabor et al., 1980; Whittington et al., 1983] and subsequent serologic testing [Schlauder et al., 1995] indicated that the GB agent was distinct from the other hepatitis viruses (i.e., A, B, C, D, and E). The isolation of two novel RNA viruses associated with GB agent has been reported recently [Simons et al., 1995]; however, the method used to isolate these viruses did not identify immunogenic regions of virus-encoded proteins. The nonspecific amplification technique was therefore employed to produce an cDNA expression library which could be immunoscreened using convalescent serum from GB-agent infected tamarins. The virus source for these experiments was pooled, infectious tamarin serum (12 ml, containing approximately 10^5 infectious doses/ml) that was centrifuged onto a cesium chloride cushion in order to concentrate virus. RNA isolated from 20% of this material (equivalent to 2.4×10^5 infectious doses) was converted to cDNA and subsequently amplified nonspecifically. Approximately 300,000 recombinants from the resulting λgt11 cDNA library were immunoscreened using convalescent serum from a GB agent-infected tamarin. Three unique immunoreactive clones were isolated. Sequence analysis of the cDNA inserts revealed that they encoded portions of the GB virus B RNA genome [Simons et al., 1995] corresponding to the NS3, NS5A, and NS5B regions [Muerhoff et al., 1995].

Nonspecific amplification techniques provide a means for the isolation of sequences from very small amounts of source material, and, when coupled with standard immunoscreening methods, allow for the identification of immunogenic regions of novel viruses. Thus, while these methods have made the process of virus-specific sequence isolation more efficient, they encounter difficulty when the amount of background sequences are high, such as when utilizing tissues from infected individuals as the source of viral nucleic acids, or if the volumes of antisera are limiting. These problems can be circumvented if a subtractive procedure is utilized to diminish the complexity of the nucleic acid mixture (i.e., remove host-derived sequences) prior to PCR amplification and cloning.

Subtractive-based Approaches for the Isolation of Unique Nucleic Acid Sequences

The advent of subtractive technologies has simplified greatly the cloning of specific unknown sequences from large, complex mixtures of nucleic acids. In general, subtraction is based on removing common sequences while leaving behind sequences that differ between two complex sources of nucleic acid. Such a process can be applied to any pair of nucleic acid sources—for example, treated versus untreated cells or tissues, diseased versus normal tissue, different strains of the same organism, or tissues or blood obtained prior to and after infection of an experimental animal with a potentially infectious substance. When two highly similar nucleic acid samples are analyzed, the nucleic acid derived from the untreated or control source is referred to as the "driver," while that isolated from the treated or experimental source is referred to as the "tester." Following subtraction of tester with the driver nucleic acids, the "difference products" should, in theory, contain those sequences that are differentially expressed (cDNA subtraction), or, in the case of genomic DNA subtraction, those sequences that have been deleted. More recent approaches combining subtractive technologies with gene amplification have further advanced the field by allowing the isolation of sequences from extremely low amounts of nucleic acids.

Subtractive technologies were first introduced in the mid-1960s [Bautz and Reilly, 1966], and since then have been modified numerous times. For the most part, the subtractive component has remained the same, i.e., a large excess of double-stranded driver DNA is allowed to hybridize with single-stranded tester DNA for a given period of time. At the conclusion, single stranded nucleic acid is isolated from the double-stranded component. The rationale behind the procedure is that sequences held in common between tester and driver will anneal and this double-stranded nucleic acid can be separated from the remaining "unique" single-stranded material. The likelihood that sequences common to tester and driver will hybridize is enhanced by the driver being present in large excess. Often this subtraction procedure is repeated several times to maximize the enrichment of unique sequences. The original method of separating single- from double-strand nucleic acids utilized hydroxylapatite chromatography [Affara and Daubas, 1979; Timberlake, 1980]. Single-strand nucleic acids isolated by this method were often used as enriched probes to screen cDNA libraries. Compared with differential screening of cDNA libraries, the use of enriched probes meant increased sensitivity and thus more rapid identification of the sequence of interest.

Modifications to the subtraction procedure have been directed toward separation of double-stranded from single-stranded nucleic acid. Such modifications have included the use of biotinylated driver DNA for efficient removal of double-stranded nucleic acid by streptavidin affinity chromatography and/or phenol:chloroform extraction [Welcher et al., 1986]; and the use of oligo-dT-latex particles to produce cDNA from a driver source. The latex-cDNA is then hybridized to mRNA from the tester source followed by removal of annealed sequences via centrifugation, thus leaving behind

unique, single-stranded mRNA [Hara et al., 1991]. Frequently, these modifications resulted in greater enrichment of target sequences; however, only the most abundant sequences are most likely to be recovered by these procedures. Thus, sequences that are expressed differentially, but only to a minor degree, often go undetected. Unique sequences recovered by subtractive technologies include genes expressed in specific organisms [Welcher et al., 1986], senescent tissues [Hara et al., 1993], diseased versus normal tissues [Austruy et al., 1993; Schraml et al., 1993; Schweinfest et al., 1990], and developmentally regulated genes [Rothstein et al., 1993; Sive and St. John, 1988].

A further significant advancement in the technology was the addition of gene amplification following subtraction. Amplification was made possible by adding unique sequences to the ends of all nucleic acids either by ligation [Wieland et al., 1990] or by DNA synthesis [Hara et al., 1993]. Because all DNAs then have homologous ends, primer-specific amplification is made possible regardless of the intervening sequences which are unknown. The advantage of gene amplification is that it allows the recovery of gene sequences present in the smallest quantities following subtraction; in addition, it permits the use of very low amounts of starting material than are typically required for traditional subtraction methods.

Representational Difference Analysis

Representational difference analysis (RDA) is the latest modification to differential cloning methodologies, simultaneously combining subtraction with gene amplification in order to isolate difference products from two complex mammalian genomes [Lisitsyn et al., 1993]. RDA accomplishes this feat by reducing the genome complexity through restriction endonuclease digestion of the genomic DNA and subsequent PCR amplification following the addition of specific linkers to the resulting DNA fragments (Fig. 2). During PCR amplification, the smaller DNA fragments tend to be amplified most efficiently; thus the resulting amplicons are made up of only a representative collection of DNA restriction fragments. This collection may represent only 10% of the genome. Though a minority of the genome is present in a single restriction enzyme representation, multiple overlapping representations can be made by using different restriction enzymes, allowing the genome to be broadly encompassed.

After obtaining the restriction enzyme representations of the tester and driver, a second set of specific linkers are added only to the tester. Tester and driver representations are denatured and allowed to anneal in the presence to excess driver, followed by PCR amplification. Only those annealed fragments where both strands are derived from the tester are amplified in an exponential fashion; the remainder are amplified linearly or not at all. Sequences common to both tester and driver are thus eliminated after several rounds of subtraction and amplification. The amplified fragments obtained are in a clonable form allowing further

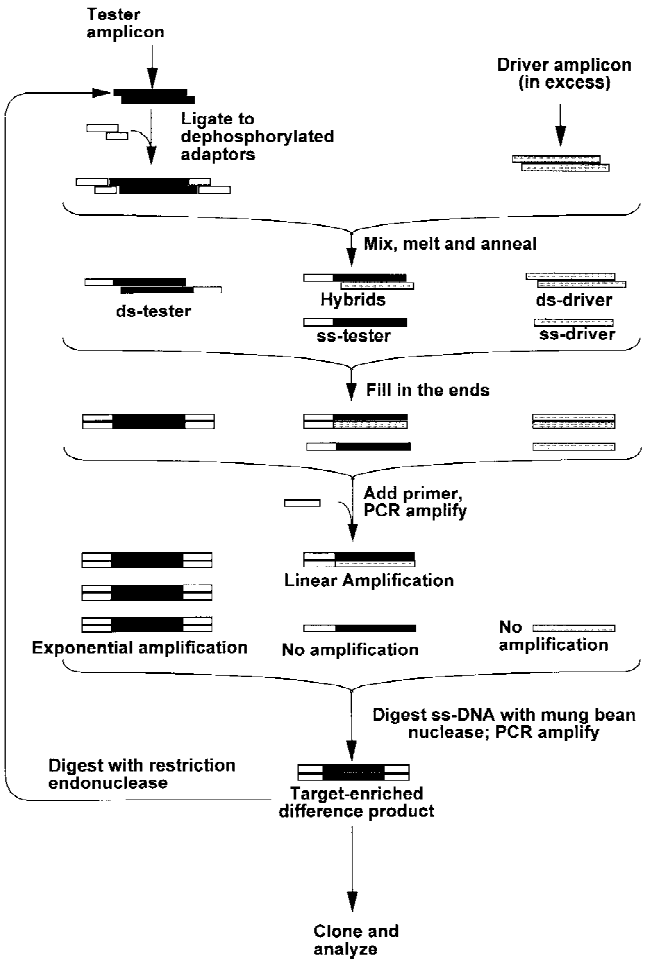

Fig. 2. Diagrammatic representation of the RDA procedure, illustrating the hybridization and amplification steps following the generation of the representative amplicons. Tester amplicons are shown as filled bars and driver amplicons as shaded bars; primers are shown as short, open bars. This subtraction/amplification procedure can be repeated several times, using the newly enriched tester amplicon as the starting material for each subsequent round. Upon sufficient enrichment, the tester amplicons can be cloned for library construction, used as a probe source for library screening, or sequenced directly [Lisitsyn et al., 1993].

analysis. The major limitation to RDA is that two highly matched nucleic acid sources are required for efficient recovery of difference products; thus source materials must be chosen carefully.

In a relatively short period of time, a number of significant discoveries have been made using the RDA methodology. A gamma herpes virus that is the putative cause of Kaposi's sarcoma lesions [Chang et al., 1994] and the GB hepatitis agent that had eluded researchers for thirty years [Simons et al., 1995] were isolated recently by RDA. Additionally, this technology has recently been used to demonstrate the presence of human herpes virus 6 in neural lesions from patients with multiple sclerosis [Challoner et al., 1995]. Studies are currently under way in each of these cases to establish a causal role for these viruses in the disease states. The identification of genetic markers appears to be another valuable role for RDA. Recently, markers

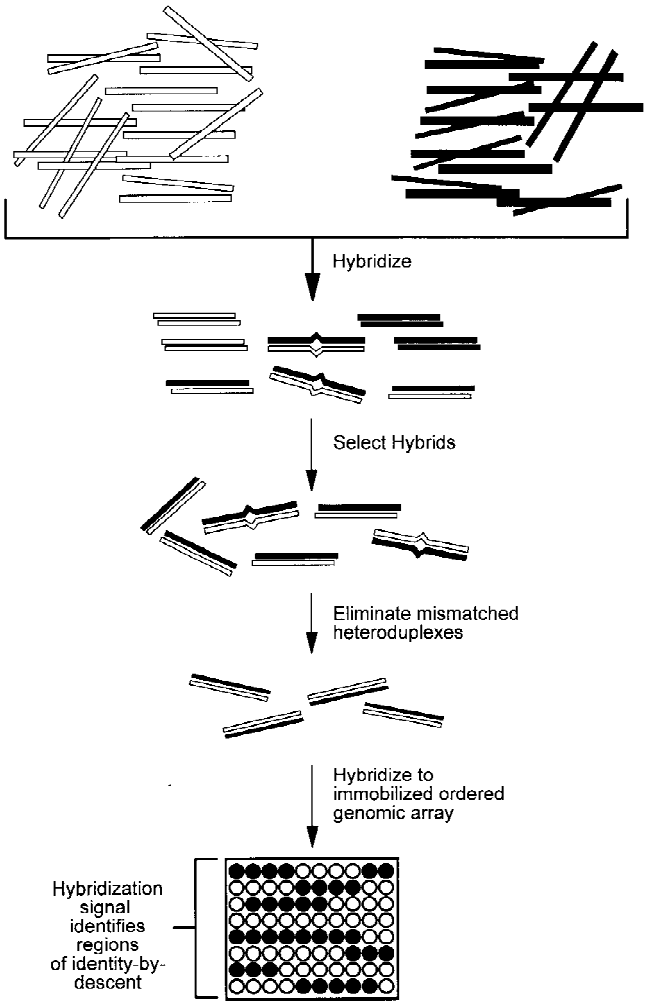

Fig. 3. Genomic mismatch scanning (GMS). Genomic DNA from two sources are digested with the same restriction enzyme. One of the samples is then treated with the *E. coli* Dam methylase while the other DNA sample remains unmethylated. Following solution hybridization, treatment of the duplex molecule population with methylation-sensitive endonucleases and purified enzymes of the *E. coli* mismatch repair system removes hybrids containing base-pair mismatches. The hybrids remaining are those which possess a high degree of sequence similarity—that is, they are assumed to be identical-by-descent. These heterohybrids are then hybridized to an ordered genomic array to identify the region of the genome from which the sequences are derived.

for three distinct polymorphisms in nude mice have been found [Lisitsyn et al., 1994]. RDA could be invaluable in the identification of markers for a number of human diseases and novel DNA and RNA viruses.

Genome Mismatch Scanning

Another subtraction method that was developed recently is known as genomic mismatch scanning [Nelson et al., 1993]. Genome mismatch scanning (GMS) is essentially the opposite of RDA: it identifies regions of identity between two genomes as opposed to regions of difference (Fig. 3) [Brown, 1994]. This method was developed originally in order to make linkage mapping of human disease genes much faster and less labor intensive. Its goal is to identify genomic sequences that are identical between two related individuals, preferably from the same family, who suffer from the same inherited disorder. Genomic regions that exhibit identity-by-descent between the individuals will presumably be linked closely with the disease causing gene or genes.

Genome mismatch scanning utilizes mismatch repair enzymes to recognize base mismatches between DNA heteroduplexes formed following solution hybridization of DNA from two individuals. If the heteroduplexes are sufficiently large, then there is a high degree of certainty that mismatches will exist between allelic sequences that are not identical-by-descent. The process requires that the DNA from one individual be methylated using an enzyme isolated from *E. coli.* Following hybridization, heteroduplex molecules (composed of one strand of DNA from each individual) of complete identity are isolated by selective elimination of heteroduplexes that contain mismatches through the use of methylation-sensitive endonucleases and purified enzyme components of the *E. coli* mismatch repair system. The fragments isolated by this procedure are then used to probe an ordered array of DNA fragments or clones representing the entire genome. Those fragments that are identical-by-descent will hybridize to the same DNA fragments within the ordered array. These DNA fragments can then be mapped more finely or can be sequenced directly, depending upon their size. This method has been successfully applied to yeast but has not yet been extended to the human genome.

In theory, GMS could be utilized for virus discovery, although this application has not been promoted by its inventors. To apply GMS to virus isolation one would isolate total nucleic acids derived from two unrelated patients believed to be infected with the same unknown infectious agent. The use of unrelated patients would be beneficial in this case as they are less likely to possess genomic sequences that are identical-by-descent. Thus, following completion of the GMS procedure, the remaining sequences would include those derived from the infectious agent, since this is the sequence that would be held in common between the two individuals. The virus-derived sequences could then be identified by using the GMS-selected fragments to hybridize against ordered arrays of total nucleic acid sequences (clones) from both infected and uninfected individuals. It would be necessary to hybridize to nucleic acid arrays derived from both individuals, since the only way to identify the virus-derived sequence is by differential hybridization. In the ideal situation, the GMS-selected fragments would be made up entirely of virus-derived sequences, and therefore, only DNA fragments from the infected individual should hybridize to the probe. These fragments could then be sequenced directly. This application of the GMS process, however, has some major drawbacks. First, infectious agents are notorious for their ability to mutate rapidly, especially RNA viruses such as HIV and HCV. Thus, comparing the total nucleic acids isolated from two unrelated in-

dividuals may contain sequence variants of the etiologic agent which would be eliminated during the procedure. Second, the construction of complete nucleic acid libraries from each individual would be very expensive and time consuming. Third, the GMS-selected fragments would most likely contain not only the virus-derived sequences but also other sequences of identity, making the interpretation of the differential hybridization results more complicated. These roadblocks, and others related to the problem of dealing with a genome as complex as that of humans, do not make GMS as attractive for use in virus discovery as RDA, which identifies differences (i.e., presence versus absence of the infectious agent) between complex nucleic acids mixtures as opposed to similarities.

Other Amplification Methods

In addition to the procedures described above, other amplification methods have been described recently that could be utilized for virus discovery. These are nucleic acid-based amplification (NASBA) and differential display. Nucleic acid-based amplification or NASBA [Compton, 1991; Guatelli et al., 1990], in its original form, utilizes gene-specific primers, reverse transcriptase, T7 RNA polymerase, and RNase H in an isothermal amplification of dsDNA target molecules. This method would require modification of the cDNA synthesis step to allow for the all nucleic acid species isolated from the infectious source to be amplified non-specifically. In this manifestation the procedure would parallel SISPA or NSA, as described above, but with NASBA substituting for PCR amplification. The NASBA technique has been applied to the amplification-detection of specific sequences, i.e., for in vitro diagnostic evaluation of bodily fluids for the presence of papilloma virus [Smits et al., 1995], HIV [Bush et al., 1992], hepatitis B [Feinman et al., 1992], and hepatitis C [Lunel et al., 1995]. The method is particularly well suited for the detection of single- or double-strand RNA viruses, since one of the enzymes utilized is reverse transcriptase. The attractive feature of NASBA is its lack of requirement for thermal denaturation for amplification and thus does not require specially designed instrumentation. However, it has not been applied to the generation of complex mixtures of nucleic acids for subsequent cloning, screening, or subtraction procedures, although this is certainly a potential application of the process. Other isothermal amplification procedures have been described, such as strand-displacement amplification (SDA) [Walker et al., 1992a; Walker et al., 1992b] and transcription-mediated amplification (TMA) [Boothroyd et al., 1995; Fultz and Kacian, 1996], but they do not necessarily possess any advantages over amplification methods utilizing thermostable polymerases for the amplification of nucleic acids. SDA and TMA have been applied to the diagnosis of infectious diseases.

Another technique with a direct application to virus discovery is differential display or RNA-arbitrarily primed PCR (RAP-PCR). This method allows the identification of genes that are expressed differentially in various cells or by the same cell under different conditions [Liang and Pardee, 1992; Welsh et al., 1992]. The differential display method described by Liang and Pardee utilizes a 3′-end "locking" primer containing a random sequence followed by an oligo-dT sequence for the production of cDNA transcripts from poly-A+ mRNA. The resultant cDNAs are then amplified arbitrarily by using a short sense primer of 9–10 nucleotides in length and an antisense primer corresponding to the 3′-end locking primer. The use of multiple primer sets allows one to obtain a fingerprint of RNA transcripts that are differentially expressed. The RAP-PCR method described by Welsh et al. [1992] utilizes a non-anchor primer-dependent method of cDNA synthesis and also utilizes PCR protocols and primers that minimize the amplification of genomic DNA. Following amplification, the products, which will vary in size, depending upon the relative positions of primer annealing, are separated by electrophoresis on a DNA sequencing gel. Products that are shown to be expressed in the "induced" condition are then excised from the gel, re-amplified, and sequenced. Either method can be applied directly to the process of virus discovery, given that any replicating virus will produce RNA transcripts encoding viral proteins. The RAP-PCR method has the advantage over classical differential display in that it does not require polyadenylated mRNA. The nucleic acid source for such experiments can consist of serum or plasma, isolated cells, cultured cells, or tissues from infected and uninfected individuals, preferably from experimental animals prior to and after inoculation with the putative infectious material. As with the other mentioned amplification techniques, an advantage of differential display is the small amounts of starting material required.

Summary

The use of PCR amplification of nucleic acids sequences permits investigations when only small amounts of source material are available. In addition, the methodologies described permit the possible identification of an etiologic agent from potentially infectious samples when suitable cell culture capabilities for virus propagation or animal models are not yet available. The use of amplification-based methods for virus discovery has the potential to accelerate the identification of novel viruses or virus strains responsible for human disease and thus provide more rapid diagnosis and eventual treatment.

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